The experimental model of acute gastritis such as water immersion restraint (WRS) stress-induced gastric injury is useful tool in examination of pathomechanism of acute gastritis. Nitric oxide (NO) plays an important role in the maintenance of gastric barrier, however, the interaction between reactive oxygen species (ROS) and NO on gastric mucosal integrity has been little studied. The purpose of our present study was to explain the participation of ROS in healing of WRS-induced gastric lesions accelerated by NO. Experiments were carrying out on 120 male Wistar rats.

To assess gastric blood flow (GBF) laser Doppler flowmeter was used and the number of gastric lesions was counted in each stomach. The colorimetric assays were used to determine gastric tissue level of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the products of lipid peroxidation by ROS, as well as superoxide dismutase (SOD) activity, the enzyme scavanger of ROS. We demonstrated that 3.5 h of WRS resulted in appearance of acute gastric lesions accompanied by a significant decrease of GBF. Biological effects of ROS were estimated by measuring tissue levels of MDA and 4-HNE, as well as the SOD activity. It was demonstrated that 3.5 h of WRS led to significant increase of mucosal levels of MDA and 4-HNE, and it was accompanied by a decrease of SOD activity. Pretreatment with NO-donors (SIN-1, SNAP, nitroglycerin, NO-ASA) resulted in reduction in gastric lesion number, increment of GBF, decrease of MDA and 4-HNE tissue level and increase of SOD activity. Suppression of ROS plays an important role in the action of NO-donors on healing of acute gastric lesions induced by 3.5 h of WRS. NO-donors caused an attenuation of lipid peroxidation as documented by a decrease of MDA and 4-HNE levels and enhancement of antioxidative properties as evidenced by an increase of SOD activity.

Key words: water immersion restraint stress, MDA, SOD, NO-donors, SIN-1, SNAP, nitroglycerin, NO-ASA
INTRODUCTION

Acute gastric mucosal lesions represent an important clinical problem. Animal models allow to recognize details of pathomechanisms of acute gastric damage. The model of water immersion and restraint stress (WRS), proposed by Takagi et al. (1), is especially useful in these investigations. This model appears to be very suitable in testing various factors affecting the formation and healing of gastric mucosal lesions, for example sulphhydrals, endogenous prostaglandins (2), growth factors, polyamines (3), etc. The effect of apoptosis on production of gastric lesions in this model has also been determined (4). Experiments on the role of gastric acid secretion and certain bacterial metabolites, affecting this secretion such as N-alpha-methylhistamine in acute WRS ulcerogenesis, were carried out (5).

The gastric barrier protects the mucosa against damage of its deeper structures by hydrogen ion and other noxious substances originating from the gastric lumen (6). An important role in damage and protection of this barrier is played by gastric microcirculation. Disturbances in blood perfusion of gastric mucosa result in the formation of erosions and ulcers. This phenomenon typically occurs in experimental model of ischemic gastric lesions (7).

The main factors, which regulate gastric blood flow, are prostaglandins, sensory peptides released from endings of afferent nerves and nitric oxide (8, 9). The function of nitric oxide (NO) in the regulation of gastric blood flow (GBF), which participates in maintenance of mucosal integrity, has been a subject of many investigations (10-12). These investigations focused on the involvement of NO in protection of gastric mucosal perfusion as well as on the interaction of NO and prostaglandins on gastric mucosa.

Little information is available regarding the contribution of reactive oxygen species (ROS) and NO to the mechanism of gastric mucosal integrity. Previous studies focused mainly on the participation of ROS in experimental ischemic model of gastric lesions (13, 14). Results of our previous experiments indicated that 3.5 hours of WRS leads to increased oxidative metabolism, comparable with that observed in ischemia-reperfusion model of gastric injury. Under these conditions the evaluation of ROS participation in the NO action on gastric mucosal seems to be of interest. Two parameters are usually useful for assessment of biological effects of ROS namely the tissue levels of malondialdehyde (MDA) plus 4-hydroxynonenal (4-HNE) and the activity of superoxide dismutase (SOD). Tissue levels of MDA and 4-HNE are used as indicators of lipid peroxidation. SOD activity reflects the antioxidative properties of various tissues including gastric mucosa (Fig. 1).

In our experiments acute gastric lesions have been induced by 3.5 hours of WRS and intragastrical administration of NO-donors was used prior to the WRS. The aim of our present investigations was to elucidate the participation of ROS in healing of WRS-induced gastric lesions, in tests without and with addition of NO-donors.
MATERIAL AND METHODS

Experiments were carried out on 120 male Wistar rats, weighing about 200 g and fasted for 24 h before all studies. Studies were approved by the Ethic Committee for Animal Research of Jagiellonian University College of Medicine.

Production of gastric lesions

The animals were divided into 6 groups. In first group, rats underwent 3.5 h of WRS in temperature 23°C, using the method originally proposed by Takagi et al. (1). In second group, 3-morpholinosyndnoimine (SIN-1) was administered intragastrically (i.g.), using a metal orogastric tube in a dose of 5 mg/kg, given 30 min prior to 3.5 h of WRS. In third group S-nitroso-N-acetyl-DL-penicillamine (SNAP) was applied intragastrically (i.g.) in dose of 5 mg/kg, 30 min prior to 3.5 h of WRS. In forth group nitroglycerin (10 mg/kg i.g.) was used 30 min prior to 3.5 h of WRS. In fifth group NO-aspirin (NO-ASA) - 40 mg/kg i.g. - was administered 30 min prior to 3.5 h of WRS. Group sixth of animals served as a control group and did not undergo any procedures (control group).

Determination of gastric blood flow and number of lesions

The gastric lesions were evaluated 3.5 hours after start of WRS. To assess gastric blood flow (GBF) laser Doppler flowmeter (LaserFlo, model BPM 403A, Blood Perfusion Monitor, Vasamedics, St. Paul, Minnesota, USA) was used. The animals were anaesthetized with Vetbutal 50 mg/kg (Biowet, Pu³awy, Poland), then the abdomen was opened and the stomach was exposed to determine the GBF. Blood flow was measured on anterior and posterior wall of stomach. The mean values of these measurements were calculated and expressed as percent change from value recorded in intact mucosa. To establish the number of gastric lesions computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany) was used, as described previously (5, 15).

Fig. 1. Transformation pathways of a superoxide radical anion in the organ tissue. Superoxide is metabolized via the process of lipid peroxidation or neutralization undergo to form H₂O₂ and H₂O.
Measurement of lipid peroxidation

For determination of lipid peroxidation in tested groups, tissue levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured and they were used as indicators of lipid peroxidation. The procedure of MDA and 4-HNE determination was as follows: about 600 mg of gastric mucosa was excised, quickly washed in test tube and 20 ml 0.5 M BHT (butylated hydroxylanluene) was added in order to prevent sample oxidation. This sample was subsequently homogenized in 20 mM Tris for 15 sec. in pH 7.4. Then homogenate was centrifuged (3000 g at 4°C for 10 min) and obtained clear supernatant was stored at -80°C prior to testing.

The colorimetric assay for lipid peroxidation (Bioxytech LPO-586, Oxis, Portland, USA) was used to determine of MDA and 4-HNE tissue concentration. This assay is based on the reaction of a chromogenic reagent N-methyl-2-phenylindole with MDA and 4-HNE at 45°C. This reaction yields a stable chromophore with maximal absorbance at 586 nm. This absorbance was measured by spectrophotometer Marcel s300, Warsaw, Poland. Results were expressed as nanomol per gram of tissue (nmol/g) according to our study published previously (15).

Determination of SOD activity

For the determination of activity of superoxide dismutase (SOD), a sample of gastric mucosa was taken, as described above. The colorimetric assay for assessment of SOD activity (Bioxytech SOD-525, Oxis, Portland, USA) was used. This method is based on the SOD-mediated increase in the rate of autooxidation of tetrahydrobenzofluorene in aqueous alkaline solution to yield a chromophore.

![Graph](image)

Fig. 2. Mean number of gastric lesions and gastric blood flow (GBF) after 3.5 h of water immersion restraint stress (WRS) without or with intragastrical pretreatment (i.g.) with SIN-1 (5 mg/kg i.g.) or SNAP (5 mg/kg i.g.). Results are mean ± SEM of 6-8 rats. Cross (+) indicates significant change, as compared with the value obtained in vehicle control group. Asterisk (*) indicates significant change, as compared with the values obtained in rats exposed to 3.5 h of WRS alone.
with maximum absorbance at 525 nm. This absorbance was measured by spectrophotometer Marcel s300, Warsaw, Poland. Results were expressed as units per gram of tissue (U/g)(15).

Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was done using nonparametric Mann-Whitney test. Differences with P<0.05 were considered as significant.

RESULTS

Gastric lesions and gastric blood flow

Figs 2 and 3 show means of lesion number in the WRS model as well as gastric blood flow (GBF). Intact mucosa (control) did not show any macroscopic lesions and the GBF in this intact mucosa averaged 46±5 ml/min/100 g of tissue and this value was considered as basal flow (100%). Following 3.5 h of WRS numerous acute gastric lesions occurred and the mean lesion number was about 24±1.5, while the GBF was reduced to 43±5 % of control value. Pretreatment with SIN-1 (5 mg/kg i.g.) and SNAP (5 mg/kg i.g.) resulted in a significant reduction of gastric lesion number and these effects were accompanied by an

![Graph showing gastric lesions and GBF](image)

Fig. 3. Mean number of gastric lesions and gastric blood flow (GBF) in rats exposed to 3.5 h of water immersion restraint stress (WRS) without or with intragastric pretreatment (i.g.) with nitroglycerin (10 mg/kg i.g.) or NO-aspirin (NO-ASA 40 mg/kg i.g.). Results are mean ± SEM of 6-8 rats. Cross (+) indicates significant change, as compared with the respective values in control group. Asterisk (*) indicates significant change, as compared with the respective values in rats subjected to 3.5 h of WRS.
increase of GBF as compared with the 3.5 h of WRS group. After administration of SIN-1 at a dose of 5 mg/kg (Fig. 2) a decrease of mean lesion number to 6.6±1.2 was observed. Administration of nitroglycerin (10 mg/kg i.g.) or NO-ASA (40 mg/kg i.g.) evoked similar effects to those observed with SIN-1, namely a reduction in gastric lesion number to the values of 14.0±1.0, 13.7±1.6, respectively, and a significant rise in the GBF (Fig. 3).

**Lipid peroxidation products**

Concentration of MDA and 4-HNE in the intact mucosa was very low, near to analytical limit of detection (5.9±0.05 nmol/g). After 3.5 h of WRS the level of MDA and 4-HNE increased almost three times, reaching the value of 15.8±1.0 nmol/g. Administration of SIN-1 (5 mg/kg i.g.) resulted in a significant decrease in MDA and 4-HNE concentrations as compared to respective values in animals exposed to the 3.5 h of WRS alone. Application of SNAP (5 mg/kg i.g.) produced significant decrease of MDA and 4-HNE levels, as compared with those observed in the 3.5 h of WRS group (Fig. 4). Pretreatment with nitroglycerin (10 mg/kg i.g.) or NO-ASA (40 mg/kg i.g.) led to significant fall in lipid peroxide.
metabolites, as compared with those in rats exposed to 3.5 h of WRS alone. The value of MDA and 4-HNE mucosal concentration reached minimum in animals treated with nitroglycerin prior to WRS (Fig. 5).

The MDA results in all investigated groups were significantly higher, as compared with the respective values obtained in the intact mucosa (Figs 4, 5).

**SOD activity**

In intact gastric mucosa (control group) SOD activity averaged about 347.8±58.0 U/g. Following exposure of rats to WRS, a significant decrease of SOD activity (245.2±22.0 U/g) was observed. Intragastrical administration of SIN-1 or SNAP, applied in the doses of 5 mg/kg, resulted in a significant rise of SOD activity, as compared to that observed with WRS (Fig. 6). Similar increase in SOD activity was observed after pretreatment with NO-ASA (40 mg/kg i.g.). Significant increase of SOD activity, as compared with that in WRS mucosa, was demonstrated when nitroglycerin (10 mg/kg i.g.) was used. After pretreatment with nitroglycerin, SOD activity reached maximal level (600.3±10.1 U/g) (Fig. 7).

![Diagram](image_url)  
*Fig. 5. Concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the gastric mucosa of rats exposed to 3.5 h of water immersion restraint stress (WRS) without or with intragastric (i.g.) pretreatment with nitroglycerin (10 mg/kg i.g.) or NO-aspirin (NO-ASA 40 mg/kg i.g.). Results are mean ± SEM of 6-8 rats. Cross (+) indicates significant increase, as compared to the value obtained in the control group. Asterisk (*) indicates significant decrease, as compared with the values obtained in group exposed to WRS alone.*
Nitric oxide (NO) plays an important regulatory role in maintaining gastric mucosal integrity. Previous studies focused mainly on NO as a gastroprotective factor, that is released in large quantities in the mucosa and contributes to the reduction of the area and number of gastric lesions. These studies attempted to explain the mechanisms of NO in cell protection and for this purpose the substrate of NO synthesis (L-arginine) or inhibitors of NO synthase (e.g. L-NNA, L-NAME) were used (16-19). Cooperation between NO and prostaglandins in gastroprotection was described (20). Previous studies using experimental stress model, emphasized the importance of NO, released from capsaicin-sensitive afferent nerves, in the interaction with epidermal growth factor (EGF) on healing of WRS-induced gastric lesions (21). However, little information is available regarding the participation of reactive oxygen species (ROS) in NO-induced protection and accompanying increase of GBF.

Until now, ROS were reported to play a deleterious role in pathomechanism of gastric mucosal injury. Pohle et al. (22) demonstrated that ROS are involved in aspirin-induced gastric lesions in humans, where treatment with radical
scavengers prevented the NSAID-induced gastropathy. The ROS mediated lipid peroxidation was proposed by Naito et al. (23) to be a major cause of indomethacin-induced gastric lesions. In addition, ROS and afferent C-fibers have been proposed to play an important role in the pathomechanisms of thermal stress (24). It was suggested that the ulcerogenesis depends upon the interplay between ROS generation and NO action (14). In another study NO was recognized as a factor which prevents oxidative stress, for example by the inhibition of leukocyte adherence (25).

Our previous studies documented that ROS are involved in the formation of gastric mucosal damage due to an enhancing effect on lipid peroxidation and attenuation of mucosal antioxidant mechanisms (15). This notion is in keeping with our present observations that the production of ROS contribute to the pathomechanisms of stress-induced gastric lesions. This is why we determined the effect of NO-donors on ROS generation in rats exposed to 3.5 h of WRS.

Mechanism of protective action of NO-donors appears to be multifactorial. Classic theories may include the vasodilatation, caused by NO-donors, which ultimately leads to the increase in organ perfusion (26-28). Besides gut NO

![Fig. 7. Superoxide dismutase (SOD) activity in the gastric mucosa of rats exposed to 3.5. h of water immersion restraint stress (WRS) without or with intragastric (i.g.) pretreatment with nitroglycerin (10 mg/kg i.g.) or NO-aspirin (NO-ASA 40 mg/kg i.g.). Results are mean ± SEM of 6-10 rats. Cross (+) indicates a significant increase, as compared with the value obtained in control group. Asterisk (*) indicates a significant decrease, as compared with the value obtained in control group. Double cross (++) indicates a significant change as compared with the respective value in rats exposed to WRS alone.](image-url)
released from SIN-1 exerts anti-atherogenic properties by alteration of LDL metabolism in macrophages (29). SNAP, another NO donor, strongly influences the cardiovascular regulation in hypertension (30). Previous studies revealed that NO-donors accelerated healing of acute and chronic gastric ulcerations. Nitroglycerin attenuated mucosal damage of ethanol through counteracting effect of this NO-donor on the ethanol-induced fall in potential difference (PD) across the stomach wall (31). The gastroprotective properties of SNAP depend upon the inhibition by this NO-donor of gastric acid secretion that has been documented in isolated parietal cells (32). Moreover SNAP, injected intraperitoneally prevented ethanol-induced gastric lesions and this protection was accompanied by increased gastric blood flow (33).

NO-ASA, despite the inhibition of cyclooxygenase (COX)-activity protects the gastric mucosa against damage induced by strong irritants and enhances healing of gastric ulcers due to release of NO (34, 35). It was proposed that NO, which is released from NO-ASA, compensates for the deficiency of prostaglandin synthesis induced by this NSAID (36). Fiorucci et al. (37, 38) demonstrated that NO-ASA exhibits sparing effect on gastric mucosa by inhibition of apoptosis and attenuation of proinflammatory cytokines such as TNFα and IL-1β. In another report Takeuchi et al. (39) showed protective activity of NO-ASA in a model of thermic stress. NO-donors can exert an opposite effect after their administration in high doses. For instance the application of SNAP in high dose delayed healing of ethanol-induced gastric lesions, as demonstrated previously. (33).

So far little information is available regarding the effect of NO-donors on ROS formation in the gastric mucosa. We demonstrated recently that NO-ASA, in contrast to native ASA, failed to delay the healing of chronic gastric ulcer and this effect was accompanied by the attenuation of lipid peroxidation (40). Wallace et al. (41) showed that NO-ASA had inhibitory effect on neutrophil adherence to the vascular endothelium. This was attributed to the decrease in neutrophil infiltration of gastric mucosa, resulting in attenuation of oxidative tissue damage. As mentioned above, NO can also be considered as a pathogenic factor contributing to tissue damage. Konaka et al. (42) observed intensification of lipid peroxidation and myeloperoxidase activity, during indomethacin-induced small intestinal lesions in rats, the effect being associated with the increased NO production in the intestinal tissue.

Our present study was designed to determine the effect of NO-donors such as SIN-1, SNAP, nitroglycerin, NO-ASA on the lesions induced by WRS. We found that NO-donors reduced gastric lesions and that this protection was accompanied by the fall in oxidative stress parameters, namely decrease of MDA and 4-HNE levels and increase of SOD activity to the level observed in the intact mucosa. The most prominent suppression of lipid peroxidation was observed in nitroglycerin-treated gastric mucosa while maximal increase of SOD activity occurred when SNAP was administered prior to WRS exposure. Thus, we conclude that gastroprotective mechanism of SNAP involves suppression by this
agent of ROS generation and concomitant increase in SOD activity, that appears to protect the gastric mucosa from oxidative damage induced by WRS.

In conclusion our present results demonstrated the beneficial role of NO, released from SIN-1, SNAP, nitroglycerin and NO-ASA, in gastroprotection against WRS damage. We also determined that the role of NO-induced protection depends upon the attenuation of lipid peroxidation and involves the restoration of SOD activity in the gastric mucosa subjected to stress.

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